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(54) Title: METHOD OF IDENTIFYING INDIVIDUALS SUSCEPTIBLE TO MYOCARDIAL INFARCTION, A NEED FOR CORONARY SURGERY, AND STROKE BY DETECTING THE LEVELS OF FACTOR IX ACTIVATION PEPTIDE

Second Northwick Park Heart Study

Factor IX activation peptide

	n	mean	SD	P
Coronary events+	103	252.0	148.8	0.02*
Stroke	20	244.2	90.6	0.19*
All controls	264	215.9	78.6	

+ Clinical and silent myocardial infarction plus coronary surgery

(57) Abstract

Levels of Factor IX activation protein (FIXP) can be used to identify individuals who are susceptible to myocardial infarction, the need for coronary surgery, and stroke. Any method of detection of FIXP can be used, preferably radioimmunoassays, preferably utilizing monoclonal antibodies to FIXP.

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METHOD OF IDENTIFYING INDIVIDUALS SUSCEPTIBLE TO MYOCARDIAL INFARCTION, A NEED FOR CORONARY SURGERY, AND STROKE BY DETECTING THE LEVELS OF FACTOR IX ACTIVATION PEPTIDE

This application claims priority under 35 U.S.C. 119(e) to the provisional U.S. application Serial No. 60/101,310 entitled "Method of Identifying Individuals Susceptible to Myocardial Infarction, a Need for Coronary Surgery, and Stroke by Detecting the Levels of Factor IX Activation Peptide Use of Growth Factors and Hormones for Expansion of Mammalian Cells and Tissue Engineering," by Bauer et al., filed Sept. 21, 1998 and hereby incorporated in its entirety by reference.

Background of the Invention

Heart disease and stroke are leading causes of death. An obstacle to successful prevention of these causes hasbeen development of reliable means for the identification of those at higher risk of coronary heart disease (heart attack) and cerebrovascular disease (stroke). A goal of medical treatment is timely intervention to reduce risk. Research has shown association between high cholesterol levels and coronary disease, while high blood pressure and smoking raise the risks of heart attack and stroke. However, these three major risk factors, as they are called, are linked to the underlying disorder of the arteries in these two disorders, atherosclerosis. What causes heart attach and stroke is not atherosclerosis per se, but super-added thrombosis (blood clot) blocking the diseased artery. Many patients with atherosclerosis do not develop thrombotic complications. Hence a majority of patients with raised cholesterol, high blood pressures and who smoke, do not develop either

premature heart attack or stroke. What has been needed in order to impose prediction are markers equivalent to cholesterol which predict high risk of thrombosis. The present invention has now identified such a marker, which is called factor IX activation peptide. Factor IX is one of the blood clotting factors which, when activated, is mounted to a clotting enzyme with release of a small non-functional peptide, factor IX activation peptide (FIXP).

Summary of the Invention

The present invention provides a method of identifying individuals who are susceptible to a coronary event or a stroke by detecting the levels of activation of factors in the coagulation cascade and subsequently comparing levels to a "control group". These factors include but are not limited to factors IX, X, XII and XII and related peptides. In addition the present invention provides a method of identifying individuals who are susceptible to a coronary event or a stroke by detecting the levels of Factor IX activation peptide. For this application, a coronary event is defined as any clinical myocardial infarctions or heart attacks, "silent" myocardial infarctions as detected by electrocardiogram, and coronary surgery including, but not limited, to coronary bypass and angioplasty. A control group is defined as a group of healthy individuals matched for age, locality and time when examined where healthy is defined as an individual who had not experienced a myocardial infarction or stroke or a need for coronary surgery. Methods of detection include any method, direct or indirect, of measuring levels of factor IX activation peptide.

The method of detection of factor IX activation peptide for correlation to a coronary event indicating an increase of approximately one standard deviation units above the average

for a control group comprising healthy individuals who have not suffered a coronary event is associated with a 54% increase in risk for a coronary event. The correlation is highly statistically significant (p=0.01). The same increase of one standard deviation units above the average for diagnosing susceptibility to stroke correlates with an approximately 174% increase in risk with a slightly lower degree of statistical significance (p=0.08).

Definitions

P is probability of difference in two data sets occurring by random sampling from single data set (i.e. probability that there is no real difference in the quantities being measured from two data sets. See Shoemaker et al. *Experiments in Physical Chemistry. Chapter II. Treatment of Experimental Data. Significance Testing*. pp. 35-38. Incorporated herein by reference.)

Coronary event includes clinical and silent myocardial infarction and coronary surgery and any thromobotic events. Coronary surgery includes but is not limited to coronary bypass surgery and angioplasty.

Control group are healthy individuals matched for age, locality and time when examined where healthy is defined as an individual who had not experienced myocardial infarction or stroke or a need for coronary surgery.

RIA is radioimmunoassay.

Description of the Drawings

Figure 1. Table from the Second Northwick Park Heart Study. Levels of Factor IX Activation Peptide. Data are in pmol/L.

Figure 2. Table from the Second Northwick Park Heart Study. Odds ratio for a one Standard Deviation increase in the level of Factor IX activation peptide. Measure of risk for a coronary event and for stroke correlated with the level of factor IX activation peptide. Data are in pmol/L.

Description of Certain Preferred Embodiments

Blood Coagulation

(from Bauer et al. Blood 78(4): 731-736, 1990 which is incorporated herein by reference).

The coagulation system of blood has classically been described as consisting of two distinct pathways, the intrinsic and extrinsic cascades which converge to activate factor X. The initiation of the intrinsic system is thought to occur with damage to the endothelium and the resulting exposure of subendothelial components such as collagen to the blood. The components of the intrinsic mechanism include the contact factors (factor XI), prekallikrein, high molecular-weight kininogen, factor XI as well as factors IX and VIII. The extrinsic pathway is initiated when factor VII binds to tissue factor, an integral membrane glycoprotein that is expressed constitutively by subendothelial components of the vessel wall. The factor Xa that is generated by either of the two cascades is then able to convert prothrombin to

thrombin by binding to factor Va on activated platelets. The action of thrombin upon fibrinogen results in the formation of a fibrin clot.

During the last 35 years, data have accumulated indicating that the intrinsic and extrinsic coagulation mechanisms do not function independently of each other. In the 1960s several groups of investigators provided circumstantial evidence that the components responsible for the initiation of the extrinsic cascade could activate factor IX in plasma. However, it was not until 1977 that Osterud and Rapaport (Proc.Natl.Acad.Sci.USA 74:5260, 1977) clearly demonstrated that a mixture of partially purified factor VII and tissue factor could activate purified factor IX.

Human factor IX is a single-chain vitamin K-dependent glycoprotein with a mol wt of 57,000 that circulates in plasma as an inactive zymogen at a concentration of ~70 nmol/L. The entire amino-acid structure of the human protein has been deduced from the cloning of its cDNA. The activation of this component by factor XIa or the factor VII tissue factor complex leads to the formation of factor IXa, which consists of two polypeptide chains joined by a disulfide bridge. During these reactions, peptide bonds at Arg_{145} Ala_{140} and Arg_{150}-Val_{151} are cleaved, which releases a highly glycosylated 35 amino-acid activation peptide with a mol wt of ~ 11,000.

There are currently no data regarding the relative contributions of the contact system and the factor VII-tissue factor mechanism to factor IX a generation in vivo. Bauer et al. (Blood 78(4): 731-736, 1990) describe the development of the radioimmunoassay RIA for the activation peptide that is released from factor IX upon activation by factor VII tissue-factor complex. The application of this technique to the study of patient populations with severe

hereditary deficiencies of factor XI and factor VII indicates that the factor VII-tissue factor mechanism contributes in a dominate fashion to factor IX activation in humans.

Detection of Factor IX Activation Peptide

The present invention provides a method of identifying individuals who are susceptible to a coronary event or a stroke by detecting the levels of Factor IX activation peptide and comparing levels to a control group of healthy individuals. In addition, detection can involve detection of activation of any factor in the coagulation cascase. The step of detecting Factor IX activation peptide can be any method of detecting the levels of factor IX activation peptide. Without limitating the scope of the invention, these methods include any method utilizing any molecules or reagents that are capable of associating with factor IX activation peptide, or capable of associating with transcript encoding Factor IX activation peptide.

Alternatively or additionally, detection of levels of Factor IX activation peptide and activation of any factor in the coagulation cascase includes detecting levels of factors that are responsible for production of Factor IX activation peptide or factors involved in activation of any factor involved in the coagulation cascase (F XIa or F VII tissue factor complex.).

Other methods of detecting individuals who are susceptible to a coronary event or a stroke include but are not limited to, detection of DNA polymorphisms associated with DNA sequences that control the production of factor IX activation peptide such as promotor and enhancer sequences. These include but are not limited to DNA sequences encoding factor IX, factor XIa or factor VII tissue factor and related regulatory sequences. Also, detection

of activators or repressors of the gene encoding Factor IX and factor IX activation peptide can be utilized. Other methods of detecting Factor IX activation peptide include but are not limited to detecting levels of transcription and translational regulators of factor IX activation peptides, detection of regulators of factor IX activation peptide stability, proteases of factor IX, factor IX activation peptides and regulators thereof and mRNA encoding factor IX, factor IX activation peptides and regulators thereof.

Where detection is by means of a molecules that associates with factor IX activation peptide, the associating agent can be any molecule or material. The association can be direct or indirect and by any means of association such covalent, hydrophobic and van der Waals interactions. Alternatively, in preferred embodiments, the associating agent is a peptide preferably an antibody, most preferably a monoclonal antibody. The associating agent can be a small molecule (as used herein, the term "small molecule" refers to an organic compound either synthesized in the laboratory or found in nature. Typically, a small molecule is characterized in that it contains several carbon-carbon bonds, and has a molecular weight of less than 1500 grams/Mol), or a nucleic acid. Associating agents can be naturally occurring or synthesized.

The method of detection can also utilize, without limitation to the present invention, radioactive markers, chromogenic markers, chemiluminescent markers, fluoresence markers, and any luminescent markers.

In one particularly preferred embodiment, immunobased assays such as ELISA (enzyme-linked immunosorbent assay) are utilized to detect levels of factor IX activation peptide. In a particularly preferred embodiment, a method of detecting factor IX activation

peptide utilizes a double antibody assay where the two antibodies are associated with fluorescence tags. The fluorescent tags are chosen such that when in close proximity, one tag can quench the fluorescent emissions of the other tag. Alternatively, the fluorescent tags can be chosen such that when in close proximity, the fluorescent emission from one tag can excite the other tag to fluoresce at a difference wavelength indicating proximity of the two tags.

Where the detection method involves detection of factor IX activation peptide itself, the protein is preferably purified from plasma using the method described in Bauer et al. (Blood 78(4): 731-736, 1990). The method of processing of plasma for purification of factor IX activation peptide for RIA is described in Example 2.

The present invention will now be illustrated by the following Examples which are not meant to limit the scope of the invention. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains. The following examples are intended to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the novel methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention in any way.

Example 1

Second Northwick Park Heart Study

To identified IX peptide as a marker of risk of heart attack and stroke, about 3000 healthy men, initially free of heart disease or a history of stroke, were examined and blood taken for measurement of several clotting factors including IX peptide. They were followed

on average for 6 years, during which time all heart attacks and strokes were recorded by regular inspection of primary care, hospital and coroner's records. A blood sample for each participant was stored at - 80°C to permit analysis for IX peptide at the end of the study in all men who developed heart attack or stroke, and to compare their results with those of a sample of men who remained healthy, matched for age, locality and time when examined.

By 1st January, 1998 there had been 71 heart attacks, 15 men needing coronary bypasses or angioplasty for threatening heart attack, 18 men with new evidence of "silent" heart attack on their electrocardiograms, 20 strokes, and 9 sudden death of uncertain cause. Table 1 shows data comparing the concentrations of factor IX activation peptide in three groups of individuals (those suffering from coronary events, those suffering from a stroke, and a control group of healthy individuals.)

P=0.02 for the group suffering from coronary events versus the control group indicates a 2% probability that the difference in the mean number is not significant (random fluctuation of data from one data set.). Conventionally accepted probability level for considering differences to be significant is 5% or less (Shoemaker et al.)

Table 2 indicates the odds ratio for a difference between the group suffering from a coronary event or suffering from a stroke versus the control group. The odds ratio of 1.54 for coronary events means a 54% increase in risk for a one standard deviation increase in Factor IX activation peptide (the 95% confidence limits are a 10% increase to a 116% increase). The probability (P) of 0.012 indicates that a difference of about 100 units (?) of factor IX activation peptide in a group suffering from a coronary event versus a healthy

control group was associated with a 54% increase in the risk of a coronary event with a 1.2% statistical probability of the risk being statistically insignificant.

For the group suffering from strokes versus the control group, the data are interpreted with the same method. An odds ratio of 2.74 indicates a 174% increase in risk for a one standard deviation increase in the level of Factor IX activation peptide. However, note that the lower confidence limit is a 12% decrease as compared to the data for the group suffering from a coronary event. Hence, a probability of (p) = 0.08. (the SD in this case/control study is about 90).

1) For clinical and silent heart attack combined, plus coronary artery surgery, an increase of about 100 units above the average was associated with a 54% increase in risk of one of the above. This was highly significant (p = 0.01).

2) For stroke, the same increase in IX peptide appeared to be associated a more than doubling of risk, though because of small numbers this finding was not quite statistically significant (p = 0.08).

Thus, factor IX activation peptide promises to be a useful marker of risk of thrombosis in middle-aged men.

Factor IX activation peptide is obtained from blood samples as described by Bauer et al. (Blood 78(4): 731-736, 1990 which is incorporated herein by reference). Example 2 describes the revised protocol for processing plasma for factor IX activation peptide for RIA.

Example 2

Protocol for Processing of plasma for Factor IX activation peptide RIA.

The protocol for the preparation of Factor IX activation peptide from plasma for RIA is essentially as described by Bauer et al (Blood 78(4): 731-736, 1990) except for the following modifications as indicated.

The antibody fractions used in the FIXP RIA are approximately 1000-fold less reactive toward factor IX than the activation peptide. Thus a normal plasma factor IX level of 70 nmol/L might contribute as much as 0.07 nmol/L to the immunoreactive signal. We also observed that plasma constituents other than FIXP or factor IX contributed to a nonspecific basal signal in the assay. It was therefore necessary to develop a method by which the peptide could be extracted from plasmas from individual subjects. In this procedure larger proteins were precipitated by adding 0.1 vol of 7 mol/L perchloric acid to 0.9 vol of plasma and were then removed by centrifugation at 48,000 g for 20 minutes at room temperature. The supernatant fluid was decanted 5 N NaOH was added to raise the pH to greater than 7 and 40% (as compared to 20% for Bauer et al) (vol/vol) trifluoroacetic acid (TFA) was admixed to lower the pH to less than 3. A butylsilane C_4 (6 mL) extraction column (J.T. Baker Inc. Philipsburg, NJ) was prepared with 6 mL of absolute methanol containing 0.5% (vol/vol) TFA followed by 6 mL of 0.5% TFA. The sample was applied to this column which was then washed with 12 mL of 0.5% TFA. The peptide was subsequently eluted with 4 mL of 75% (vol/vol) methanol containing 0.5% TFA into 12 x 75 mm test tubes. The contents of the tubes were evaporated to dryness overnight in a Savant Speed Vac Concentrator. Samples were individually reconstituted with 1.5 mL of 0.10

mol/L NaCl in 0.05 mol/L Tris-HCl pH 8.0 (as compared to 7.5 in Bauer et al.) containing 0.02% (wt/vol) sodium azide and 1 mg/mL ovalbumin. The specimens were then assayed by RIA for FIXP immunoreactivity.

The extraction of 3 mL (as compared to 5 mL in Bauer et al) of plasma from individual subjects enables us to concentrate the FIXP signal fourfold and lowers the detection limit of the assay technique to 5 pmol/L. A normal plasma control "spiked" with 0.5 pmol of FIXP as well as the same control plasma are included with each set of samples to be processed. The results obtained on 10 occasions with a plasma pool obtained from normal donors indicate that the uncorrected basal level of the activation fragment is 211 $\pm$ 14 pmol/L. The recovery of the added peptide averaged 84.4% $\pm$ 9.9%. The extent of recovery was independent of the amount of FIXP added to the plasma or the volume of plasma used in the extraction process. As we have found that the average recovery of added FIXP in the extraction procedure is essentially constant we have elected to not divide the values obtained by the fractional recovery. While the intra-assay coefficient of variation for the FIXP RIA was less than 5%, the within-day and between-day coefficients of variation for a plasma sample subjected to our extraction procedure were 8% and 12% respectively.

Claims

We claim:

1. A method of diagnosing susceptibility to coronary events comprising the steps of:

providing a sample of blood from an individual;

detecting a level of factor IX activation peptide; and

comparing the level to a control group wherein said control group consist of healthy individuals with similar gender, age, race, without coronary events.

2. A method of diagnosing susceptibility to myocardial infarction comprising the steps of:

providing a sample of blood from an individual;

detecting a level of factor IX activation peptide; and

comparing the level to a control group wherein said control group consist of healthy individuals with similar gender, age, race, without coronary events.

3. A method of diagnosing susceptibility to stroke comprising the steps of:

providing a sample of blood from an individual;

detecting a level of factor IX activation peptide; and

comparing the level to a control group wherein said control group consist of healthy individuals with similar gender, age, race, without coronary events.

Figure 1

Second Northwick Park Heart Study

Factor IX activation peptide

	n	mean	SD	P
Coronary events+	103	252.0	148.8	0.02*
Stroke	20	244.2	90.6	0.19*
All controls	264	215.9	78.6	

+ Clinical and silent myocardial infarction plus coronary surgery

* versus all controls.

Figure 2

Second Northwick Park Heart Study

Factor IX activation peptide (IX pep)

	Odds ratio for a one SD increase in IX pep	P
Coronary event+	1.54 (1.10, 2.16)	0.012
Stroke	2.74 (0.88, 8.55)	0.082

+ Clinical and silent myocardial infarction plus coronary surgery

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(74) Agent: JARRELL, Brenda, H.; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).

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(54) Title: DIAGNOSIS OF MYOCARDIAL DISEASE OR STROKE USING FACTOR IX ACTIVATION PEPTIDE

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* ... all subjects.

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Levels of Factor IX activation protein (FIXP) can be used to identify individuals who are susceptible to myocardial infarction, the need for coronary surgery, and stroke. Any method of detection of FIXP can be used, preferably radioimmunoassays, preferably utilizing monoclonal antibodies to FIXP.

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INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOLOGICAL ABSTRACTS Philadelphia PA USA; abstract no. PREV199699034109; abstract XP002132112 & G.J. MILLER ET AL.: "Increased activation of the haemostatic system in men at high risk of fatal coronary heart disease" THROMBOSIS AND HAEMOSTASIS, vol. 75, no. 5, 1996, pages 767-771, Berlin FRG — -/—	1,2

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MEDLINE, Washington DC USA; abstract no. 98189523, abstract XP002132113 & G.J. MILLER ET AL.: "Activation of the coagulant pathway in cigaret smokers" THROMBOSIS AND HAEMOSTASIS, vol. 79, no. 3, 1 March 1998 (1998-03-01), pages 549-553, Berlin FRG	3

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